PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 25 August 1999 (25.08.99)	
International application No. PCT/AU99/00069	**Applicant's or agent's file reference** 83547
International filing date (day/month/year) 01 February 1999 (01.02.99)	**Priority date** (day/month/year) 02 February 1998 (02.02.98)
Applicant EXNER, Thomas	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

05 August 1999 (05.08.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

[illegible]rnational Bureau of WIPO [illegible]emin des Colombettes [illegible]eva 20, Switzerland [illegible].35	Authorized officer Lazar Joseph Panakal Telephone No.: (41-22) 338.83.38

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/00069

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: G01N 033/86, C12Q 001/56

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N 033/86, 033/96; C12Q 001/56

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU: IPC as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT, JAPIO, MEDLINE, KEYWORDS:
VENOM, AKISTRODON, CONTORTRIX, FACTOR Xa, CLOTTING, COAGULATION

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DE 3724443 A (MAX PLANK GES WISSENSCH) 2 February 1989 Whole document	1-21
A	AU 69785/87 A (BOEHRINGER MANNHEIM GMBH) 10 September 1987 Whole document	1-21
A	AU 31059/85 A (GRADIPORE LTD) 15 February 1996 Page 6 line 19 to page 7 line 7 and claims	1-21

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 February 1999	23 FEB 1999

Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	ROSS OSBORNE Telephone No.: (02) 6283 2404

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/00069

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 91/01382 A (KABIVITRUM) 7 February 1991 Page 13 line 29 - page 14 line 19.	1-21
A	Thromb. Haemost, 1997 Mar, 77(3) pp 436-9, Armando Tripodi et al, Screening for the FV: Q506 Mutation - evaluation of thirteen plasma. based methods for their diagnostic efficacy in comparison with DNA analysis. Page 436-437 (methods section) and page 438 column 2 lines 1-5	1-21
A	Thrombosis Research 1997 87(6) pp 501-510 Katharina Ruzicka et al, Evaluation of a new screening assay Proc® Global for identification of defects in the protein C/protein S anti coagulant pathway. Abstract	1-21

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU 99/00069

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
DE	3724443	NONE					
AU	69785/87	CA	1299075	DE	3607559	DK	1181/87
		EP	236985	JP	62212569	NO	870945
		US	5001069				
AU	31059/85	CA	1313025	EP	332324	JP	1307428
		NO	890943	NZ	228183	US	4946660
		ZA	8901595				
WO	9101382	AU	60533/90	CA	2063564	EP	486515
		NO	920125	SE	8902532	US	5439802

END OF ANNEX

PATENT COOPERATION TREATY

From the:
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

F B Rice & Co
605 Darling Street
BALMAIN NSW 2041

PCT

WRITTEN OPINION

(PCT Rule 66)

	Date of mailing *(day/month/year)* 07 DEC 1999
Applicant's or agent's file reference 83457	**REPLY DUE** within **ONE MONTH** from the above date of mailing

International application No.	International filing date *(day/month/year)*	Priority Date *(day/month/year)*
PCT/AU 99/00069	1 February 1999	2 February 1998

International Patent Classification (IPC) or both national classification and IPC

Int. Cl.[6] G01N 33/86, 33/96, C12Q 1/56

Applicant

GRADIPORE LTD et al

1. This written opinion is the **second** (first, etc) drawn by this International Preliminary Examining Authority.
2. This opinion contains indications relating to the following items:.

I	[X]	Basis of the opinion
II	[ ]	Priority
III	[ ]	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	[ ]	Lack of unity of invention
V	[X]	Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	[ ]	Certain documents cited
VII	[ ]	Certain defects in the international application
VIII	[X]	Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

When? See the time limit indicated above. The applicant may, before the expiration of that time limit, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also For an additional opportunity to submit amendments, see Rule 66.4. For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4*bis*. For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: **2 June 2000**

Name and mailing address of the IPEA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. (02) 6285 3929	Authorized Officer **ROSS OSBORNE** Telephone No. (02) 6283 2404

WRITTEN OPINION

International application No.
PCT/AU 99/00069

I. Basis of the opinion

1. With regard to the **elements** of the international application:*

☐ the international application as originally filed.

☒ the description, pages **1,8, 10-12, 14-16** as originally filed,
pages , filed with the demand,
pages **2-7,9, 13** filed with the letter of **27 October 1999** .

☒ the claims, pages **18-19** as originally filed,
pages , as amended under Article 19,
pages , filed with the demand,
pages **17** filed with the letter of **26 October**.

☒ the drawings, pages **1/5-5/5,** as originally filed,
pages , filed with the demand,
pages , filed with the letter of .

☐ the sequence listing part of the description:
pages , as originally filed
pages , filed with the demand
pages , filed with the letter of .

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language which is:

☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the written opinion was drawn on the basis of the sequence listing:

☐ contained in the international application in printed form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☐ The amendments have resulted in the cancellation of:

☐ the description, pages

☐ the claims, Nos.

☐ the drawings, sheets/fig.

5. ☐ This opinion has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed"*

WRITTEN OPINION

International application No.
PCT/AU 99/00069

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims **1-21**	**YES**
	Claims	**NO**
Inventive step (IS)	Claims **1-21**	**YES**
	Claims	**NO**
Industrial applicability (IA)	Claims **1-21**	**YES**
	Claims	**NO**

2. Citations and explanations

Claims 1-21. The invention is a method of determining coagulation potential of a plasma sample.

None of the citations disclose the combination of features that includes:

1) Preincubation of the plasma sample with a protein C activator and factor Xa, where the Xa is progressively inactivated

and

2) The use of dilute Agkistrodon venom or like reagent.

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Claims 1-4 and 7-21 are not fully supported by the description. The description provides no substantive disclosure of any suitable exogenous protein C activators other than dilute whole Agkistrodon venoms. It is the finding that there is a surprising result in using dilute Agkistrodon venoms in the coagulation assay that is the core of the invention and there is no reasonable basis provided for the extension to other agents when the basis of the effect is not known. The dilution required also needs to be defined so that the scope of the claims is made clear.

Our Ref: 83457

25 October 1999

IP Australia - Patent Office
International Preliminary Examining Authority
PO Box 200
WODEN ACT 2606

GRADIPORE LIMITED, THOMAS EXNER
International Patent Application No PCT/AU99/00069
Entitled: "Improved blood coagulation test"

Dear Sir

DUE DATE FOR RESPONSE: 30 OCTOBER 1999

In response to the Written Opinion dated 30 August 1999 the applicants hereby offer the following comments and enclosed substitute page(s) 3, 4 and 17 under Article 34(2).

The differences between the pages on file and the attached substitute page(s) are:

Page 3: Mention of "step (b)" at lines 24 and 26 have been amended to "step (c)".

Page 3: At lines 34-35, the words "exogenous reagent which transforms protein C into activated protein C" has been amended to read "reagent used in step (a), which transforms protein C into activated protein C,".

Page 17: In claim 1, mention of "step (b)" in the comparison step (d) have been corrected to "step (c)".

Claim 4: The words "the exogenous reagent which transforms protein C into activated protein C is" have been amended to read "the reagent used in step (a) comprises".

... /2

IP Australia - Patent Office
Our Ref: 83457

25 October 1999

At Box VIII of the Written Opinion, it has been stated that claims 1-4 and 7-21 are not fully supported by the description since there is allegedly "no substantive disclosure of any suitable exogenous protein C activators other than the dilute whole Agkistrodon venoms". In response, we wish to refer the IPEA to, for example, page 3 line 34 to page 4 line 1 and page 4 lines 18-32, where it is clearly disclosed that suitable protein C activators include components from venoms from species related to *Agkistrodon Contortrix*. Further, while admittedly producing poorer results than dilute whole Agkistrodon snake venom, the commercial protein C activator known as PROTAC™ is still suitable for use in the present invention. Thus, the present specification makes it abundantly clear that the use of dilute whole snake venom in the preincubation reagent is preferred only. We therefore respectfully submit that claims 1-4 and 7-21 are clearly supported by the present specification.

Also at Box VIII of the Written Opinion, it has been stated that claims 4-6 are unclear in their dependency to claims 1-3. In response, an amendment has been made to claim 4 in order to clarify that the mentioned reagent is that which is used in the preincubation step (a).

Finally, at Box VIII of the Written Opinion, it has also been stated that the term "blood coagulation potential" is not fully defined. In response, we respectfully submit that persons skilled in the art would readily understand what is meant by this term. In particular, persons skilled in the art would understand that "blood coagulation potential" refers to the capacity of a blood sample to clot more or less quickly or to produce a larger or smaller amount of fibrin clot. Thus, a high blood coagulation potential (or "hypercoagulability") refers to the capacity of a blood sample to clot more quickly than what is normally observed, and is associated with high levels of clotting factors, low levels of antithrombotic proteins and a high risk of thrombosis. On the other hand, a low blood coagulation potential refers to a poor or defective capacity of a blood sample to clot. It is therefore respectfully submitted that the IPEA's statements concerning the term "blood coagulation potential" are made wholly without foundation.

In view of the above, we respectfully request reconsideration and withdrawal of the negative comments concerning the present claims.

Yours respectfully
F B RICE & CO

MOD/rcn/j21/pat049
Encl

such as heparin sulphates normally on endothelial cells lining healthy blood vessels.

The present inventor has made the surprising finding that such tests may be further modified to allow improved discrimination between healthy individuals and patients with impaired or aberrant blood anti-thrombotic mechanisms.

Disclosure of Invention

In a first aspect, the present invention consists in a method of determining the coagulation potential of a plasma sample, the method comprising the steps of:

(a) preincubating the plasma sample with a reagent such that

(i) endogenous protein C in the plasma is at least partially converted into activated protein C by the reagent, and

(ii) adding factor Xa which is progressively inactivated by antithrombin III/heparin cofactor 2 during the preincubation;

(b) adding to the preincubated plasma sample (a) reagents to initiate clotting comprising:

(i) an exogenous reagent which activates factor X to Xa or prothrombin to thrombin in a factor V-dependent manner, and

(ii) components, such as phospholipid and calcium ions, that are necessary for efficient coagulation of the plasma sample;

(c) monitoring a reaction indicative of the rate of coagulation of the plasma sample;

(d) comparing the rate of coagulation detected in step (c) with the equivalent rate determined for a normal patient, or comparing the rate of coagulation detected in step (c) with the equivalent rate determined for the plasma sample in the absence of protein C activator; and

(e) determining the coagulation potential of the plasma sample from one or other of the comparisons of step (d).

The reagent used in step (a) preferably also contains low levels of glycosaminoglycans such as regular or low molecular weight heparins, dermatan or dextran sulphates in addition to factor Xa. The inclusion of these components to the reagent makes the test sensitive to antithrombin III.

Preferably, the reagent used in step (a), which transforms protein C into activated protein C, is diluted substantially whole snake venom, preferably diluted snake venom from *Agkistrodon Contortrix*, or related

species such as *A. Piscovorus, A. Bilineatus, A. C. Laticinctus, A. C. Moccason*. It has been found that by selecting an appropriate concentration of the snake venom, it is possible to obtain a diagnosis of impaired anticoagulation by the one test. A protein C pathway (PCP) ratio of below a pre-determined value can be indicative of impaired coagulation control in the patient's plasma. When using *A. Contortrix* whole venom diluted at a concentration of about 0.002%, it is possible to differentiate between plasma from normals, whether these come from healthy or pregnant or lupus anticoagulant positive individuals and plasma from individuals with thrombotic risk factors such as APC resistant factor V(Leiden) and protein C deficiency. A PCP ratio in this instance of below about 2 would be positive in the present test. Similarly for a concentration of 0.003%, a value of below 2.5 would be positive.

Preferably, the incubation in step (a) is carried out at neutral or slightly basic conditions, more preferably at about pH 7.5. The incubation is carried out for sufficient time for activation of the protein C in the plasma. Typically incubation times of around 5 minutes as usual for the preincubation interval in most automated APTT test methods have been found to be sufficient.

The present inventor has made the surprising finding that the protein C activator purified from *A. Contortrix* venom (a commercial product "Protac™" available from Pentapharm AB (Switzerland)) does not work very well in the present invention. The present inventor has found that dilute *A. Contortrix* venom is particularly suitable. It is possible that the purification process used to produce this commercial protein C activator removes an additional activator or agent that is present in whole venom which is preferably required for the present invention. The precise nature of the ingredient is not yet clear, however, it would appear to be a procoagulant unaffected by deficiency of vitamin K-dependent factors or Warfarin treatment. It will be appreciated that this additional activator or agent could also be purified from whole venom and combined with the commercially available purified protein C activator for use in the present invention. The individual active fractions may also be purified and recombined to produce a reagent suitable for the present invention.

Factor Xa of either human or animal origin can be included and incubated with the protein C activator reagent. This factor can be formed from endogenous factor X by venom activators Factor Xa tends to shorten clotting time. Thus the level of Russells viper venom which needs to be

CLAIMS:

1. A method of determining the coagulation potential of a plasma sample, the method comprising the steps of:

(a) preincubating the plasma sample with a reagent such that

(i) endogenous protein C in the plasma is at least partially converted into activated protein C by the reagent, and

(ii) adding factor Xa which is progressively inactivated by antithrombin III/heparin cofactor 2 during the preincubation;

(b) adding to the preincubated plasma sample (a) reagents to initiate clotting comprising:

(i) an exogenous reagent which activates factor X to Xa or prothrombin to thrombin in a factor V-dependent manner, and

(ii) components, such as phospholipid and calcium ions, that are necessary for efficient coagulation of the plasma sample;

(c) monitoring a reaction indicative of the rate of coagulation of the plasma sample;

(d) comparing the rate of coagulation detected in step (c) with the equivalent rate determined for a normal patient, or comparing the rate of coagulation detected in step (c) with the equivalent rate determined for the plasma sample in the absence of protein C activator; and

(e) determining the coagulation potential of the plasma sample from one or other of the comparisons of step (d).

2. The method according to claim 1 wherein the reagent used in step (a) further contains low levels of glycosaminoglycans.

3. The method according to claim 2 wherein glycosaminoglycans are selected from the group consisting of regular or low molecular weight heparins, and dermatan or dextran sulphates.

4. The method according to any one of claims 1 to 3 wherein the reagent used in step (a) comprises diluted substantially whole snake venom.

5. The method according to claim 4 wherein the diluted snake venom is derived from *Agkistrodon Contortrix*, or related species including *A. Piscovorus, A. Bilineatus, A. C. Laticinctus, A. C. Moccason*.

6. The method according to claim 5 wherein the snake venom is *A. Contortrix* whole venom diluted at a concentration of 0.002%.

A substrate conversion reaction rate may be determined by the coagulation time or by the time required for the conversion of a chromogenic substrate to a coloured product. The conversion rate obtained is compared with values obtained in the absence of protein C activator or PCA and also with results on normal plasma samples. If the coagulation time is not sufficiently prolonged by protein C activator , it indicates that the individual from which the sample is derived may be at a higher-than-normal risk of thrombosis.

It is well known that activation of endogenous protein C in plasma by the activator from *A. Contortrix* venom prolongs subsequent clotting times to a degree related to the protein C content. Several other factors, however, influence or interfere with this test. These factors include protein S, factor V(Leiden) which are now recognised as thrombotic risk factors in their own right.

The present inventor has recently developed an improved APC resistance test which is described in WO 96/04560. This test requires the addition of exogenous reagents which activate factor V and activate the common pathway of the blood coagulation mechanism through factor X or by inducing the formation of thrombin in a factor V dependant manner together with exogenous APC to a plasma sample. It was found that if factor V is specifically activated by an exogenous reagent in addition to activation of the common pathway through factor X, the test for APC resistance may be made more sensitive and specific than previously known tests. The present inventor has also found that improved specificity is obtained when a complex factor X activator is used together with the factor V activator. This test, because the Russells viper venom contains activators of both factor X and factor V, has been referred to as the Russells Viper Venom (RVV) -based test. A similar result is achieved if prothrombin is activated to thrombin by a factor V dependent activator in the presence of a factor V activator such as those from Australian elapid venoms.

The protein C pathway is one of a number of antithrombotic mechanisms operating within normal blood vessels to control coagulation and prevent thrombosis. Probably the most important of these mechanisms is the glycosaminoglycan (GAG) pathway which requires antithrombin III as a cofactor and heparin cofactor 2. Thrombin and factor Xa are controlled by these two plasma inhibitors which are modulated by glycosaminoglycans

such as heparin sulphates normally on endothelial cells lining healthy blood vessels.

The present inventor has made the surprising finding that such tests may be further modified to allow improved discrimination between healthy individuals and patients with impaired or aberrant blood anti-thrombotic mechanisms.

Disclosure of Invention

In a first aspect, the present invention consists in a method of determining the coagulation potential of a plasma sample, the method comprising the steps of:

(a) preincubating the plasma sample with a reagent such that

(i) endogenous protein C in the plasma is at least partially converted into activated protein C by the reagent, and

(ii) adding factor Xa which is progressively inactivated by antithrombin III/heparin cofactor 2 during the preincubation;

(b) adding to the preincubated plasma sample (a) reagents to initiate clotting comprising:

(i) an exogenous reagent which activates factor X to Xa or prothrombin to thrombin in a factor V-dependent manner, and

(ii) components, such as phospholipid and calcium ions, that are necessary for efficient coagulation of the plasma sample;

(c) monitoring a reaction indicative of the rate of coagulation of the plasma sample;

(d) comparing the rate of coagulation detected in step (c) with the equivalent rate determined for a normal patient, or comparing the rate of coagulation detected in step (c) with the equivalent rate determined for the plasma sample in the absence of protein C activator; and

(e) determining the coagulation potential of the plasma sample from one or other of the comparisons of step (d).

The reagent used in step (a) preferably also contains low levels of glycosaminoglycans such as regular or low molecular weight heparins, dermatan or dextran sulphates in addition to factor Xa. The inclusion of these components to the reagent makes the test more sensitive to antithrombin III.

Preferably, the reagent used in step (a), which transforms protein C into activated protein C, is diluted substantially whole snake venom,

preferably diluted snake venom from *Agkistrodon Contortrix*, or related species such as *A. Piscovorus*, *A. Bilineatus*, *A. C. Laticinctus*, *A. C. Moccason*. It has been found that by selecting an appropriate concentration of the snake venom, it is possible to obtain a diagnosis of impaired anticoagulation by the one test. A protein C pathway (PCP) ratio of below a pre-determined value can be indicative of impaired coagulation control in the patient's plasma. When using *A. Contortrix* whole venom diluted at a concentration of about 0.002%, it is possible to differentiate between plasma from normals, whether these come from healthy or pregnant or lupus anticoagulant positive individuals and plasma from individuals with thrombotic risk factors such as APC resistant factor V(Leiden) and protein C deficiency. A PCP ratio in this instance of below about 2 would be positive in the present test. Similarly for a concentration of 0.003%, a value of below 2.5 would be positive (see Figure 1).

Preferably, the incubation in step (a) is carried out at neutral or slightly basic conditions, more preferably at about pH 7.5. The incubation is carried out for sufficient time for activation of the protein C in the plasma. Typically incubation times of around 5 minutes as usual for the preincubation interval in most automated APTT test methods have been found to be sufficient.

The present inventor has made the surprising finding that the protein C activator purified from *A. Contortrix* venom (a commercial product "Protac™" available from Pentapharm AB (Switzerland)) does not work very well in the present invention (see Figure 2). The present inventor has found that dilute *A. Contortrix* venom is particularly suitable. It is possible that the purification process used to produce this commercial protein C activator removes an additional activator or agent that is present in whole venom which is preferably required for the present invention. The precise nature of the ingredient is not yet clear, however, it would appear to be a procoagulant unaffected by deficiency of vitamin K-dependent factors or Warfarin treatment. It will be appreciated that this additional activator or agent could also be purified from whole venom and combined with any commercially available purified protein C activator for use in the present invention. The individual active fractions may also be purified and recombined to produce a reagent suitable for the present invention.

Factor Xa of either human or animal origin can be included and incubated with the protein C activator reagent. This factor can be formed

from endogenous factor X by venom activators Factor Xa tends to shorten clotting time. Thus the level of Russells viper venom which needs to be present in the second reagent (with the calcium and phospholipid) to yield clotting times of 80-120 seconds on normal plasma, similar to those in regular protein C pathway tests, can be proportionally reduced. Also, heparin or glycosaminoglycans can be included in the preincubation reagent to enhance the interaction between antithrombin III and factor Xa to enhance sensitivity to low levels of antithrombin III.

In a preferred form of the present invention, the patient's plasma sample is incubated with an exogenous activator for protein C and factor X. The exogenous activator of protein C is preferably highly diluted and unfractionated *Agkistrodon contortrix* venom. The factor X activator is preferably derived from the venom of Russell viper (*Vipera Russelli*) and other immunologically cross-reactive species. The snake venoms may either be used in a diluted but unfractionated form which contributes to the simplicity of the test or, preferably, may be used in a fractionated form utilising isolated venom components.

Rather than directly activating factor X with an exogenous reagent in the second stage of such tests one may also obtain an improvement over the known activated protein C test by utilising an exogenous reagent that induces in the plasma the presence of thrombin in a factor V dependent manner. In this aspect of the invention factor V dependent prothrombin activators such as those from certain Australian *Notechis* and *Pseudonaja* venoms, such as *Pseudonaja Textilis*, *Notechis Scutatus* and *Oxyuranus Scutellatus*, may be used. The use of this system by-passes factor X and all factors above it thereby making the test more specific than that based on Russells viper venom alone. The use of additional venom-derived factor V activators is desirable exactly as described above for the Russell viper venom activated system which involves factor X activation.

In one embodiment of the invention, the components in step (b) with which the patient's plasma and its pre-incubants are to be mixed are combined into a single mixture. Such a single mixture preferably also contains supplemental components such as suitable buffers and preservatives. In addition the mixture preferably contains polybrene or another similar agent to reverse the effect of any heparin that may be present in the test samples or which may be added in the preincubation reagent (i).

The incubation mixture preferably also contains relatively high levels of phospholipid at high ionic strength to overcome non-specific inhibitors such as lupus anticoagulants that may be present in the plasma sample.

Another complicating feature in test plasma samples may be the defect caused by oral anticoagulants. Many such thrombotic patients may already be on oral anticoagulant treatment and this affects the coagulation tests currently used to assess activated protein C resistance. The conventional method for minimising such interference is by mixing test plasma with factor V deficient plasma. The present invention, however, does not necessarily require such manipulation as such antithrombotic agents if used within their therapeutic range do not necessarily adversely effect the test. The rationale behind this is apparent from Figure 1.

In another embodiment of the present invention, factor Xa may be used in the preincubation reagent at such a high level that no additional Russells viper venom may be required in the second mixed reagent (comprising then only phospholipid and calcium) to yield an ideal clotting time of 100 seconds with normal plasma (intended range of 50-200 seconds). In this case, the clotting time should be mainly affected by levels of antithrombin III (ATIII) and heparin cofactor 2 (HCF2) and not by protein C or S nor by the presence of factor V(Leiden). In this scenario, the method could be referred to as a test for the glycosaminoglycan pathway or a "GAG" test. The GAG test serves as a complimentary role to PCP tests as a preliminary screening test for likely defects in ATIII and HCF2, though in practice, it appears poorly sensitive to HCF2 (see Figure 4). This may not be a problem, however, as HCF2 in fact is of doubtful importance as a thrombotic risk factor in comparison to ATIII.

Most tests for ATIII and HCF2 presently used require a preliminary high dilution of the test plasma to be carried out. This is usually in a buffer containing high levels of heparin or GAGs to facilitate complete interaction of thrombin or factor Xa with ATIII or HCF2. The quantitative loss in thrombin or factor Xa enzyme activities is then converted to functional ATIII or HCF2 present in the test plasma. However, if a single thrombophilia screening test proves to be sensitive enough to all the known thrombotic risk factors in diagnostic practice, then an appropriate mixture of ACCV/PCA and factor Xa in the preincubation reagent and dilute Russells viper

venom/phospholipid/recalcifying reagent to provide equal sensitivity to all the known thrombotic risk factors would be preferred.

The detection system for monitoring the potential rates of change within the coagulation system may be a coagulation time assay or a chromometric or fluorometric assay using an appropriate synthetic substrate. Such detection systems are well known and described in the patent specifications referred to in the introductory portions of this specification.

Some patients' plasma may give borderline results when assayed by the method according to the present invention such that it is not possible to determine unequivocally between "normal" and factor V(Leiden) deficient plasma samples. The present inventor has made the surprising discovery that diluting these "borderline" samples with low ionic strength solutions including water and carrying out the method according to the first aspect of the present invention can differentiate between normal and factor V(Leiden) samples. The method according to the first aspect of the present invention also provides improved discrimination of FVL heterozygotes from homozygous individuals.

In a second aspect, the present invention consists in method to differentiate between patients with factor V(Leiden) from normal individuals, the method comprising diluting plasma from the patients and the normal individuals with low ionic strength solutions including water and repeating the method according to the first aspect of the present invention.

Preferably, the plasma are diluted 1:1 with water, preferably distilled or filtered water, prior to repeating the coagulation assay. The factor V(Leiden) plasma will usually have ratios equal to or less than the ratios obtained when undiluted. Furthermore, the ratios obtained for the factor V(Leiden) plasma will usually be less than the ratios obtained for normal plasma assayed with the same test conditions. Prior to the present invention, it would have been necessary to add factor V deficient plasma to all plasma test samples and then re-assay for clotting abnormalities.

The use of low ionic strength solutions, and particularly distilled water, is significantly cheaper than factor V deficient plasma that is required in tests currently used. Furthermore, low ionic strength solutions, and particularly distilled water, are far easier to source than factor V deficient plasma. The present invention therefore offers a real advantage in cost and

availability over other tests requiring factor V deficient plasma presently in use.

Brief Description of Drawings

Figure 1 shows effect of varying ACCV level in PCP/FVL tests on various test plasmas. Test plasmas preincubated for 5 minutes with varying levels of ACCV and then clotted with RVV/phospholipid/calcium reagent (LA-Confirm) in ACL300 in APTT mode. Showing RVV clotting time ratios (PCP ratios) plotted against the concentration of ACCV (%).

Figure 2 shows Protac™ or whole dilute Agkistrodon Contortrix C. venom (ACCV) dilutions were preincubated with pooled normal plasma for 5 minutes and then clotted with phospholipid-rich Russells viper venom reagent (LA-Confirm). Results show the RVV clotting times obtained on an ACL300 clotting machine plotted against the concentration of Protac™ (u/ml/20) or ACCV(ug/ml) used. (Note that PCP ratios are calculated as the RVV clotting times with any given ACCV or Protac™ level divided by the clotting time with no activator present).

Figure 3 shows effect of individual thrombotic risk factors on the PCP carried out with dilute whole ACC venom. Showing PCP ratios (RVV clotting times with and without protein C activation) plotted against level of each factor shown. From top to bottom; HCF2; ATIII; Prot.S, Prot.C, Factor V(Leiden). Mixes prepared from individual factor deficient or factor V(Leiden) positive (heterozygote) plasmas and pooled normal plasma, itself representing 100%.

Figure 4 shows effect of individual thrombotic risk factors on a mixed GAG/PCP test system. Preincubation reagent contained dilute whole ACCV and 0.002u/ml factor Xa. Reagent was mixed with each test plasma for 5 minutes at 37degC before being clotted with a reduced Russells viper venom reagent/phospholipid/calcium reagent. Results show the ratios of clotting times with and without preincubation plotted against factor level. In descending order on left axis; HCF2, Prot.S, ATIII, Prot.C, Factor V(Leiden).

Figure 5 shows borderline PCP/FVL results. Results show scatterplot of PCP/FVL ratios obtained on selected several warfarin patient with neat and water-diluted plasmas. Eight (8) neat plasmas all gave borderline abnormal ratios(1.2-1.8), but after dilution with water, improved discrimination of FVL cases from normals was achieved.

Test Procedure

Method

The PCP Test is not affected by Heparin levels of up to 0.5 IU/ml.

Test with PC Activator

1. Pre-warm a slight excess of PRVV reagent, allowing 0.1 ml per test, to 37°C ± 1°C in a reagent reservoir.
2. Dispense 0.1 ml of test plasma into a test tube.
3. Add 0.1 ml of Activator to the test plasma and warm at 37°C for 5 minutes.
4. Add 0.1 ml pre-warmed PRVV reagent and time from the moment of addition of the reagent to a clotting end-point using the tilt tube technique.
5. Repeat for duplicate test values and report the average of these as the result.

Test without PC Activator

1. Pre-warm a slight excess of PRVV Reagent, allowing 0.1 ml per test, to 37°C ± 1°C in a reagent reservoir.
2. Dispense 0.1 ml of test plasma into a test tube.
3. Add 0.1 ml of distilled water to the test plasma and warm at 37°C for 5 minutes.
3. Add 0.1 ml pre-warmed PRVV Reagent and time from the moment of addition of the reagent to a clotting end-point using the tilt tube technique.
5. Repeat for duplicate test values and report the average of these as the result.

The results of PCP tests of varying *A. Contortrix* whole venom levels on various test plasma are shown in Figure 1. The use of levels of between 0.002 to 0.004% ACCV in the test allows the differentiation between sera from normal individuals (PNP1, PNP2 and PNP3), oral anticoagulant pool (O/A pool) and pooled sera from pregnant individuals(PREG.POOL), from patients with impaired clotting function (FV(L) and FV(L)+O/A). PCP values of below 2 and 2.5, respectively for tests using 0.002 and 0.004% ACCV are seen to be indicative of impaired clotting.

Figure 1 shows the protein C pathway (PCP) clotting time ratios (clotting times with protein C activator present / those without activator)

CLAIMS:

1. A method of determining the coagulation potential of a plasma sample, the method comprising the steps of:

(a) preincubating the plasma sample with a reagent such that

(i) endogenous protein C in the plasma is at least partially converted into activated protein C by the reagent, and

(ii) adding factor Xa which is progressively inactivated by antithrombin III/heparin cofactor 2 during the preincubation;

(b) adding to the preincubated plasma sample (a) reagents to initiate clotting comprising:

(i) an exogenous reagent which activates factor X to Xa or prothrombin to thrombin in a factor V-dependent manner, and

(ii) components, such as phospholipid and calcium ions, that are necessary for efficient coagulation of the plasma sample;

(c) monitoring a reaction indicative of the rate of coagulation of the plasma sample;

(d) comparing the rate of coagulation detected in step (c) with the equivalent rate determined for a normal patient, or comparing the rate of coagulation detected in step (c) with the equivalent rate determined for the plasma sample in the absence of protein C activator; and

(e) determining the coagulation potential of the plasma sample from one or other of the comparisons of step (d).

2. The method according to claim 1 wherein the reagent used in step (a) further contains low levels of glycosaminoglycans.

3. The method according to claim 2 wherein glycosaminoglycans are selected from the group consisting of regular or low molecular weight heparins, and dermatan or dextran sulphates.

4. The method according to any one of claims 1 to 3 wherein the reagent used in step (a) comprises diluted substantially whole snake venom.

5. The method according to claim 4 wherein the diluted snake venom is derived from *Agkistrodon Contortrix*, or related species including *A. Piscovorus, A. Bilineatus, A. C. Laticinctus, A. C. Moccason.*

6. The method according to claim 5 wherein the snake venom is *A. Contortrix* whole venom diluted at a concentration of 0.002%.

A substrate conversion reaction rate may be determined by the coagulation time or by the time required for the conversion of a chromogenic substrate to a coloured product. The conversion rate obtained is compared with values obtained in the absence of protein C activator or PCA and also with results on normal plasma samples. If the coagulation time is not sufficiently prolonged by protein C activator , it indicates that the individual from which the sample is derived may be at a higher-than-normal risk of thrombosis.

It is well known that activation of endogenous protein C in plasma by the activator from *A. Contortrix* venom prolongs subsequent clotting times to a degree related to the protein C content. Several other factors, however, influence or interfere with this test. These factors include protein S, factor V(Leiden) and now recognised as thrombotic risk factors in their own right.

The present inventor has recently developed an improved APC resistance test which is described in WO 96/04560. This test requires the addition of exogenous reagents which activate factor V and activate the common pathway of the blood coagulation mechanism through factor X or by inducing the formation of thrombin in a factor V dependant manner together with exogenous APC to a plasma sample. It was found that if factor V is specifically activated by an exogenous reagent in addition to activation of the common pathway through factor X, the test for APC resistance may be made more sensitive and specific than previously known tests. The present inventor has also found that improved specificity is obtained when a complex factor X activator is used together with the factor V activator. This test, because the Russells viper venom contains activators of both factor X and factor V, has been referred to as the Russells Viper Venom (RVV) -based test. A similar result is achieved if prothrombin is activated to thrombin by a factor V dependent activator in the presence of a factor V activator such as those from Australian elapid venoms.

The protein C pathway is one of a number of antithrombotic mechanisms operating within normal blood vessels to control coagulation and prevent thrombosis. Probably the most important of these mechanisms is the glycosaminoglycan (GAG) pathway which requires antithrombin III as a cofactor and heparin cofactor 2. Thrombin and factor Xa are controlled by these two plasma inhibitors which are modulated by glycosaminoglycans

such as heparin sulphates normally on endothelial cells lining healthy blood vessels.

The present inventor has made the surprising finding that such tests may be further modified to allow improved discrimination between healthy individuals and patients with impaired or aberrant blood anti-thrombotic mechanisms.

Disclosure of Invention

In a first aspect, the present invention consists in a method of determining the coagulation potential of a plasma sample, the method comprising the steps of:

(a) preincubating the plasma sample with a reagent such that

(i) endogenous protein C in the plasma is at least partially converted into activated protein C by the reagent, and

(ii) adding factor Xa which is progressively inactivated by antithrombin III/heparin cofactor 2 during the preincubation;

(b) adding to the preincubated plasma sample (a) reagents to initiate clotting comprising:

(i) an exogenous reagent which activates factor X to Xa or prothrombin to thrombin in a factor V-dependent manner, and

(ii) components, such as phospholipid and calcium ions, that are necessary for efficient coagulation of the plasma sample;

(c) monitoring a reaction indicative of the rate of coagulation of the plasma sample;

(d) comparing the rate of coagulation detected in step (b) with the equivalent rate determined for a normal patient, or comparing the rate of coagulation detected in step (b) with the equivalent rate determined for the plasma sample in the absence of protein C activator; and

(e) determining the coagulation potential of the plasma sample from one or other of the comparisons of step (d).

The reagent used in step (a) preferably also contains low levels of glycosaminoglycans such as regular or low molecular weight heparins, dermatan or dextran sulphates in addition to factor Xa. The inclusion of these components to the reagent makes the test sensitive to antithrombin III.

Preferably, the exogenous reagent which transforms protein C into activated protein C is diluted substantially whole snake venom, preferably diluted snake venom from *Agkistrodon Contortrix*, or related species such as

A. Piscovorus, A. Bilineatus, A. C. Laticinctus, A. C. Moccason. It has been found that by selecting an appropriate concentration of the snake venom, it is possible to obtain a diagnosis of impaired anticoagulation by the one test. A protein C pathway (PCP) ratio of below a pre-determined value can be indicative of impaired coagulation control in the patient's plasma. When using *A. Contortrix* whole venom diluted at a concentration of about 0.002%, it is possible to differentiate between plasma from normals, whether these come from healthy or pregnant or lupus anticoagulant positive individuals and plasma from individuals with thrombotic risk factors such as APC resistant factor V(Leiden) and protein C deficiency. A PCP ratio in this instance of below about 2 would be positive in the present test. Similarly for a concentration of 0.003%, a value of below 2.5 would be positive.

Preferably, the incubation in step (a) is carried out at neutral or slightly basic conditions, more preferably at about pH 7.5. The incubation is carried out for sufficient time for activation of the protein C in the plasma. Typically incubation times of around 5 minutes as usual for the preincubation interval in most automated APTT test methods have been found to be sufficient.

The present inventor has made the surprising finding that the protein C activator purified from *A. Contortrix* venom (a commercial product "Protac™" available from Pentapharm AB (Switzerland)) does not work very well in the present invention. The present inventor has found that dilute *A. Contortrix* venom is particularly suitable. It is possible that the purification process used to produce this commercial protein C activator removes an additional activator or agent that is present in whole venom which is preferably required for the present invention. The precise nature of the ingredient is not yet clear, however, it would appear to be a procoagulant unaffected by deficiency of vitamin K-dependent factors or Warfarin treatment. It will be appreciated that this additional activator or agent could also be purified from whole venom and combined with the commercially available purified protein C activator for use in the present invention. The individual active fractions may also be purified and recombined to produce a reagent suitable for the present invention.

Factor Xa of either human or animal origin can be included and incubated with the protein C activator reagent. This factor can be formed from endogenous factor X by venom activators Factor Xa tends to shorten clotting time. Thus the level of Russells viper venom which needs to be

present in the second reagent (with the calcium and phospholipid) to yield clotting times of 80-120 seconds on normal plasma, similar to those in regular protein C pathway tests, can be proportionally reduced. Also, heparin or glycosaminoglycans can be included in the preincubation reagent to enhance the interaction between antithrombin III and factor Xa to enhance sensitivity to low levels of antithrombin III.

In a preferred form of the present invention, the patient's plasma sample is incubated with an exogenous activator for protein C and factor X. The exogenous activator of protein C is preferably highly diluted and unfractionated Agkistrodon Contortrix venom. The factor X activator is preferably derived from the venom of Russell viper (*Vipera Russelli*) and other immunologically cross-reactive species. The snake venoms may either be used in a diluted but unfractionated form which contributes to the simplicity of the test or, preferably, may be used in a fractionated form utilising isolated venom components.

Rather than directly activating factor X with an exogenous reagent in the second stage of such tests one may also obtain an improvement over the known activated protein C test by utilising an exogenous reagent that induces in the plasma the presence of thrombin in a factor V dependent manner. In this aspect of the invention factor V dependent prothrombin activators such as those from certain Australian *Notechis* and *Pseudonaja* venoms, such as *Pseudonaja Textilis*, *Notechis Scutatus* and *Oxyuranus Scutellatus*, may be used. The use of this system by-passes factor X and all factors above it thereby making the test more specific than that based on Russells viper venom alone. The use of additional venom-derived factor V activators is desirable exactly as described above for the Russell viper venom activated system which involves factor X activation.

In one embodiment of the invention, the components in step (b) with which the patient's plasma and its pre-incubants are to be mixed are combined into a single mixture by the use of suitable surfactants, particularly non-ionic detergents. Such a single mixture preferably also contains supplemental components such as suitable buffers and preservatives. In addition the mixture preferably contains polybrene or another similar agent to reverse the effect of any heparin that may be present in the test samples or which may be added in the preincubation reagent (i). The incubation mixture preferably also contains relatively high levels of phospholipid at

high ionic strength to overcome non-specific inhibitors such as lupus anticoagulants that may be present in the plasma sample.

Another complicating feature in test plasma samples may be the defect caused by oral anticoagulants. Many such thrombotic patients may already be on oral anticoagulant treatment and this affects the coagulation tests currently used to assess activated protein C resistance. The conventional method for minimising such interference is by mixing test plasma with factor V deficient plasma. The present invention, however, does not necessarily require such manipulation as such antithrombotic agents if used within their therapeutic range do not necessarily adversely effect the test.

In another embodiment of the present invention, factor Xa may be used in the preincubation reagent at such a high level that no additional Russells viper venom may be required in the second mixed reagent (comprising then only phospholipid and calcium) to yield an ideal clotting time of 100 seconds with normal plasma (intended range of 50-200 seconds). In this case, the clotting time should be mainly affected by levels of antithrombin III (ATIII) and heparin cofactor 2 (HCF2) and not by protein C or S nor by the presence of factor V(Leiden). In this scenario, the method could be referred to as a test for the glycosaminoglycan pathway or a "GAG" test. The GAG test serves as a complimentary role to PCP tests as a preliminary screening test for likely defects in ATIII and HCF2, though in practice, it appears poorly sensitive to HCF2. This may not be a problem, however, as HCF2 in fact is of doubtful importance as a thrombotic risk factor in comparison to ATIII.

Most tests for ATIII and HCF2 presently used require a preliminary high dilution of the test plasma to be carried out. This is usually in a buffer containing high levels of heparin or GAGs to facilitate complete interaction of thrombin or factor Xa with ATIII or HCF2. The quantitative loss in thrombin or factor Xa enzyme activities is then converted to functional ATIII or HCF2 present in the test plasma. However, if a single thrombophilia screening test proves to be sensitive enough to all the known thrombotic risk factors in diagnostic practice, then an appropriate mixture of ACCV/PCA and factor Xa in the preincubation reagent and dilute Russells viper venom/phospholipid/recalcifying reagent to provide equal sensitivity to all the known thrombotic risk factors would be preferred.

The detection system for monitoring the potential rates of change within the coagulation system may be a coagulation time assay or a chromometric or fluorometric assay using an appropriate synthetic substrate. Such detection systems are well known and described in the patent specifications referred to in the introductory portions of this specification.

Some patients' plasma may give borderline results when assayed by the method according to the present invention such that it is not possible to determine unequivocally between "normal" and factor V(Leiden) deficient plasma samples. The present inventor has made the surprising discovery that diluting these "borderline" samples with low ionic strength solutions including water and carrying out the method according to the first aspect of the present invention can differentiate between normal and factor V(Leiden) samples. The method according to the first aspect of the present invention also provides discrimination of FVL heterozygotes from homozygous individuals.

In a second aspect, the present invention consists in method to differentiate between patients with factor V(Leiden) from normal individuals, the method comprising diluting plasma from the patients and the normal individuals with low ionic strength solutions including water and repeating the method according to the first aspect of the present invention.

Preferably, the plasma are diluted 1:1 with water, preferably distilled or filtered water, prior to repeating the coagulation assay. The factor V(Leiden) plasma will usually have ratios equal to or less than the ratios obtained when undiluted. Furthermore, the ratios obtained for the factor V(Leiden) plasma will usually be less than the ratios obtained for normal plasma assayed with the same test conditions. Prior to the present invention, it would have been necessary to add factor V deficient plasma to all plasma test samples and then re-assay for clotting abnormalities.

The use of low ionic strength solutions, and particularly distilled water, is significantly cheaper than factor V deficient plasma that is required in tests currently used. Furthermore, low ionic strength solutions, and particularly distilled water, are far easier to source than factor V deficient plasma. The present invention therefore offers a real advantage in cost and availability over other tests requiring factor V deficient plasma presently in use.

Brief Description of Drawings

Figure 1 shows effect of varying ACCV level in PCP/FVL tests on various test plasmas. Test plasmas preincubated for 5 minutes with varying levels of ACCV and then clotted with RVV/phospholipid/calcium reagent (LA-Confirm) in ACL300 in APTT mode. Showing RVV clotting time ratios (PCP ratios) plotted against the concentration of ACCV (%).

Figure 2 shows Protac™ or whole dilute Agkistrodon Contortrix C. venom (ACCV) dilutions were preincubated with pooled normal plasma for 5 minutes and then clotted with phospholipid-rich Russells viper venom reagent (LA-Confirm). Results show the RVV clotting times obtained on an ACL300 clotting machine plotted against the concentration of Protac™ (u/ml/20) or ACCV(ug/ml) used. (Note that PCP ratios are calculated as the RVV clotting times with any given ACCV or Protac™ level divided by the clotting time with no activator present).

Figure 3 shows effect of individual thrombotic risk factors on the PCP carried out with dilute whole ACC venom. Showing PCP ratios (RVV clotting times with and without protein C activation) plotted against level of each factor shown. From top to bottom; HCF2; ATIII; Prot.S, Prot.C, Factor V(Leiden). Mixes prepared from individual factor deficient or factor V(Leiden) positive(heterozygote) plasmas and pooled normal plasma, itself representing 100%.

Figure 4shows effect of individual thrombotic risk factors on a mixed GAG/PCP test system. Preincubation reagent contained dilute whole ACCV and 0.002u/ml factor Xa. Reagent was mixed with each test plasma for 5 minutes at 37degC before being clotted with a reduced Russells viper venom reagent/phospholipid/calcium reagent. Results show the ratios of clotting times with and without preincubation plotted against factor level. In descending order on left axis; HCF2, Prot.S, ATIII, Prot.C, Factor V(Leiden).

Figure 5 shows borderline PCP/FVL results. Results show scatterplot of PCP/FVL ratios obtained on selected several warfarin patient with neat and water-diluted plasmas. Eight (8) neat plasmas all gave borderline abnormal ratios(1.2-1.8), but after dilution with water, improved discrimination of FVL cases from normals was achieved.

Test Procedure

Method

The PCP Test is not affected by Heparin levels of up to 0.5 IU/ml.

It is recommended that a 1:1 mix of patient plasma to factor V deficient plasma is used for testing of patients on oral anticoagulants. This will correct the factor deficiencies otherwise compromising the test.

Test with PC Activator

1. Pre-warm a slight excess of PRVV reagent, allowing 0.1 ml per test, to 37°C $\pm$ 1°C in a reagent reservoir.
2. Dispense 0.1 ml of test plasma into a test tube.
3. Add 0.1 ml of Activator to the test plasma and warm at 37°C for 5 minutes.
4. Add 0.1 ml pre-warmed PRVV reagent and time from the moment of addition of the reagent to a clotting end-point using the tilt tube technique.
5. Repeat for duplicate test values and report the average of these as the result.

Test without PC Activator

1. Pre-warm a slight excess of PRVV Reagent, allowing 0.1 ml per test, to 37°C $\pm$ 1°C in a reagent reservoir.
2. Dispense 0.1 ml of test plasma into a test tube.
3. Add 0.1 ml of distilled water to the test plasma and warm at 37°C for 5 minutes.
3. Add 0.1 ml pre-warmed PRVV Reagent and time from the moment of addition of the reagent to a clotting end-point using the tilt tube technique.
5. Repeat for duplicate test values and report the average of these as the result.

The results of PCP tests of varying *A. Contortrix* whole venom levels on various test plasma are shown in Figure 1. The use of levels of between 0.002 to 0.004% ACCV in the test allows the differentiation between sera from normal individuals (PNP1, PNP2 and PNP3), oral anticoagulant pool (O/A pool) and pooled sera from pregnant individuals(PREG.POOL), from patients with impaired clotting function (FV(L) and FV(L)+O/A). PCP values of below 2 and 2.5, respectively for tests using 0.002 and 0.004% ACCV are seen to be indicative of impaired clotting.

Figure1 shows the protein C pathway (PCP) clotting time ratios (clotting times with protein C activator present / those without activator)

CLAIMS:

1. A method of determining the coagulation potential of a plasma sample, the method comprising the steps of:

(a) preincubating the plasma sample with a reagent such that

(i) endogenous protein C in the plasma is at least partially converted into activated protein C by the reagent, and

(ii) adding factor Xa which is progressively inactivated by antithrombin III/heparin cofactor 2 during the preincubation;

(b) adding to the preincubated plasma sample (a) reagents to initiate clotting comprising:

(i) an exogenous reagent which activates factor X to Xa or prothrombin to thrombin in a factor V-dependent manner, and

(ii) components, such as phospholipid and calcium ions, that are necessary for efficient coagulation of the plasma sample;

(c) monitoring a reaction indicative of the rate of coagulation of the plasma sample;

(d) comparing the rate of coagulation detected in step (b) with the equivalent rate determined for a normal patient, or comparing the rate of coagulation detected in step (b) with the equivalent rate determined for the plasma sample in the absence of protein C activator; and

(e) determining the coagulation potential of the plasma sample from one or other of the comparisons of step (d).

2. The method according to claim 1 wherein the reagent used in step (a) further contains low levels of glycosaminoglycans.

3. The method according to claim 2 wherein glycosaminoglycans are selected from the group consisting of regular or low molecular weight heparins, and dermatan or dextran sulphates.

4. The method according to any one of claims 1 to 3 wherein the exogenous reagent which transforms protein C into activated protein C is diluted substantially whole snake venom.

5. The method according to claim 4 wherein the diluted snake venom is derived from *Agkistrodon Contortrix*, or related species including *A. Piscovorus*, *A. Bilineatus*, *A. C. Laticinctus*, *A. C. Moccason*.

6. The method according to claim 5 wherein the snake venom is *A. Contortrix* whole venom diluted at a concentration of 0.002%.